ESTROGEN AND HYPOXIA REGULATE LEVELS OF VEGF AND ITS RECEPTORS IN THE MICE UTERINE CERVIX AND ARE EXPRESSED IN HUMAN

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Abstract

ESTROGEN AND HYPOXIA REGULATE LEVELS OF VEGF AND ITS RECEPTORS IN THE MICE UTERINE CERVIX AND ARE EXPRESSED IN HUMAN

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The uterine cervix undergoes significant changes over the course of pregnancy, including increase in tissue size, pronounced microvascular remodeling and increase in local vascular endothelial growth factor (VEGF) and its receptors. VEGF has been studied in various parts of body and in various cancer cells; however the regulation of VEGF in healthy uterine cervix has not been examined. We hypothesize the most likely factor to influence VEGF in the uterine cervix may include mechanical stress, sex steroid hormones, hypoxia, relaxin and cytokines. In the present study we examined: **a**) the effects of mimicked hypoxia ($CoCl_2$) and exogenous estrogen (E_2) on the expression pattern of VEGF and its receptors, KDR and Flt-1, and **b**) the expression pattern of and identity of cell types expressing VEGF and its receptors in mice (non-pregnant) and human (non-pregnant) uterine cervices. Our present findings: **a**) show that hypoxia and E_2 induce expression of VEGF in the uterine cervix of non-diseased, non-pregnant mice; **b**) show the similarity of VEGF and its receptor (Flt-1 and KDR) expression profile in the human uterine cervix to mice uterine cervix; **c**) reveal the cellular pattern of VEGF expression and its receptors (Flt-1 and KDR), in the

uterine cervix of rodents. We conclude that VEGF mRNA and protein are altered by hypoxia and E_2 in a time or dose-dependent manner; are present and variably expressed in the uterine cervix of human and that they are synthesized by multiple cell types in both mice and human uterine cervix. Collectively, these data shed new insights into the regulation and pattern of VEGF expression in the uterine cervix of rodents and human.

Dedication

To: my husband, Keith; my son, Gabriel; mom, dad, Ayako, Ryoko, and Minori, for all the love, support and encouragement generously provided during the time of my graduate studies.

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Introduction

The uterine cervix undergoes significant changes over the course of pregnancy, including increase in tissue size, pronounced microvascular remodeling and increase in local vascular endothelial growth factor (VEGF) and its receptors. These changes are associated with increased levels of plasma estrogen (E2) (Downing and Sherwood, 1985). Outside the uterine cervix, a range of factors and conditions have been identified to influence the expression of VEGF, notably: hypoxia, cytokines [e.g., interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α)], growth factors (TGFα2, FGFs, PDGF), advanced glycation end products, vasopressor hormones, relaxin, sex steroid hormones, mechanical stress, and many more (Unemori et al., 1999; Koos et al., 2005; Taki et al., 2012; Donnelly et al., 2013).

VEGF belongs to a family of closely related growth factors, including VEGF-A, -B, -C, -D, -E and placenta growth factor (PGF) (Yan et al., 2011). VEGF-A or simply VEGF, has several biological effects and exists as several splice variants (Yan et al., 2011). These biological effects are largely mediated by two receptors (VEGFRs): kinase domain region (KDR) and fms-like tyrosine kinase-1 (Flt-1) (De Vries et al., 1992; Ferrara and Davis-Smyth, 1997). In female reproductive biology, VEGF is essential for a variety of ovarian and uterine endometrial functions that are also associated with E2, such as corpus luteum angiogenesis, mediation of cyclical growth of blood vessels and maturation of endometrium (Shibuya, 1998; Challis, 2000; Breeveld-Dwarkasing et al., 2003). VEGF has been studied in various ovarian diseases (i.e., endometriosis)(Wang et al, 2011), and in various cancer cells

(Roskoski, 2007). However, the functions of VEGF in the uterine cervix are still not fully explored, and the role of sex steroids and hypoxia in regulating the expression of VEGF and its receptors in the non-diseased uterine cervix has not been examined.

We have previously demonstrated the existence and expression profile of VEGF (164) and VEGFRs, in the uterine cervix of rodents (mice and rats); deciphered genes regulated by VEGF in the uterine cervix using DNA microarray analysis (Ferrara et al., 1998; Mowa et al., 2004); and more recently examined VEGF's specific physiological roles in cervical remodeling (CR), such as cervical epithelial cellular growth, immune cell recruitment and migration to the lumen of the uterine cervix and induction of an inflammatory response (Dussably et al., 2003; Mowa et al., 2008a; Nguyen et al., 2012).

Of these previously cited factors, the most likely to influence VEGF in the uterine cervix may include mechanical stress, sex steroid hormones, hypoxia, relaxin and cytokines, based on the following rationale: a) the growing fetus exerts increasing gravitational pressure (mechanical stress) on the uterine cervix (Zhang et al., 2009); b) there are temporal and spatial relationships between E2, relaxin, VEGF and VEGFRs during pregnancy; E2 and relaxin are known to induce robust VEGF expression in the uterus and elsewhere (Unemori et al., 1999); c) both pregnancy and infection induced by lipopolysaccharide (LPS) introduce cytokines in the uterine cervix, and LPS promotes mRNA expression of VEGF and its receptors (Dussably et al., 2003); d) if the robust growth of the uterine cervix, particularly in the last half of pregnancy, outgrows the micro-vascular network, as may be the case in cancer, it will lead to hypoxia, which is the most potent inducer of VEGF expression outside of the uterine cervix (Unemori et al., 1999; Taki et al., 2012).

In the present study, we examine: a) the effects of hypoxia and exogenous E2 on the expression pattern of VEGF and VEGFRs in non-pregnant, non-diseased mouse uterine cervix; b) details of the expression pattern and identity of cell types expressing VEGF and its receptors in mice (non-pregnant) and human (non-pregnant) uterine cervix.

Materials and Methods

Subjects used in the study

a). Mice: Non-pregnant and timed pregnant (days 11 and 17, untreated) female C57BL6/129SvEv mice, purchased from Charles Rivers, were used in the present studies. All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the local institution (Appalachian State University) and the NIH guidelines (NIH publication number 86-23). All efforts were made to minimize the number of animals used and discomfort or suffering of the animals.

b). Humans: Human uterine cervical tissues from four non-pregnant women (Sheffield, England), were also used in the present study. These tissues were harvested, fixed and processed in paraffin blocks using established standard procedures by our collaborator, Dr. Dilly Anumba (Sheffield University Medical School, Sheffield, England). The tissue samples were obtained using 8 x 5 x 5 mm cervical punch biopsies during abdominal hysterectomy (Table 1).

Animals, surgery, treatments and tissue harvest

Non-pregnant mice:

a). *Surgeries* (*Ovariectomy)*: Non-pregnant mice were ovariectomized (ovary removal), in order to prevent confounding effects of indigenous ovarian sex steroid hormones. Prior to surgery, animals were anaesthetized using a mixture of ketamine (43-129 mg/g body weight) and xylazine (8.6-26 mg/g body weight). To prevent post-surgery infections animals were administered with Baytril® antibiotic (Bayer, Leverkusen, Germany), immediately after the

surgery. Animals were then rested for seven days post-surgery, to allow complete excretion residual ovarian sex steroid hormones, confirmed during tissue extraction by significant uterine size reduction. At this time, animals with normal uterine size were eliminated from the study.

b). *Treatments:* Non-pregnant ovariectomized mice were then treated with either mimicked hypoxia (cobalt chloride, $CoCl_2$) or exogenous sex steroid hormones (17β-estradiol, E_2), to examine the impact on the VEGF and VEGFRs (KDR and Flt-1) expressions in the uterine cervix, as described below:

i). Effect of $CoCl_2$ (mimicked hypoxia) on expression of VEGF and receptors: Mice were treated with $CoCl_2$ (38.95 mg/kg body weight, dissolved in 0.9% sodium chloride, NaCl) in a time-dependent manner (30, 90 and 180 min), in order to simulate hypoxia, as described previously (Mastumoto et al., 2010). $CoCl_2$ solution was administered intra-peritoneally (IP) and intra-vaginally (intra-luminally) (IV) (n=7).

ii). E_2 administration: Mice were treated with either vehicle only (sesame oil) (negative control group) or exogenous E_2 dissolved in sesame oil, in dose dependent manner (8 mg, 4 mg and 2 mg/kg body weight, respectively, n=7), administered via IP once daily for 4 days (Mowa et al., 2008b).

c). *Tissue harvest*: Following the treatments, mice were administered with a lethal injection of sodium pentobarbital (Sleepaway®, Fort Dodge Laboratories Inc., Burlingame, CA) and perfused intra-cardially with 0.9% NaCl to flash out blood cells from the tissues. Uterine cervical tissues were then carefully dissected out under a stereomicroscope. Tissues were weighed immediately and tissues designated for Western blot analysis and real time polymerase chain reaction (real time PCR) were immediately stored at -80ºC until

processing. Tissues designated for morphological studies were fixed in 4% paraformaldehyde for minimum 24 h, followed by 0.1 M PBS for minimum 24 h at 4°C, then dehydrated with a (70%, 95% twice, 100%) for a minimum of 2 h per dilution followed by two changes of xylene 2 h each, and embedded in paraffin and stored in room temperature (RT) until sectioning.

Pregnant mice:

Untreated mice in early and late pregnancy (days 11 and 17 of gestation, respectively; *n*=4) were used to examine the cellular pattern of VEGF and VEGFRs expressions.

Mice were sacrificed as described previously; tissues were weighed immediately and stored appropriately.

Techniques Used in the Study:

a). *Basic Morphological Studies [hematoxylin (nucleus stain) and Eosin (cytoplasmic stain) staining]:* Experiments were undertaken to examine the basic histology of uterine tissues from mice (non-pregnant and pregnant) and human uterine cervix, as described here. Paraffin fixed sections were cut into 5 µm-thick sections using a microtome (Leica RM 2125 RTS, Leica microsystems, USA) and incubated at 37ºC overnight. Paraffin sections were de-paraffinized using two changes of xylene, rehydrated using serially diluted alcohol solutions (100% twice, 85%, 70% and 50%), followed by distilled water and 0.1 M PBS, for 10 min each. Tissues were stained with standard H & E staining procedure (VWR international LLC, USA) and imaged using Olympus DSU IX81 (Olympus, USA).

b). *Protein Expression Studies*: Cellular localization and tissue quantification of VEGF and VEGFRs protein expressions were studied using confocal immunofluorescence and Western blot analysis, respectively, as described below. Hypoxia inducible factor-α (HIF-α),

which is stabilized following $CoCl_2$ treatment, was used as a positive control protein for hypoxia studies:

i). Western Blot Analysis: Quantitative analysis of protein expression was performed to supplement confocal immunofluorescence data. Total protein was isolated from previously harvested frozen tissues using standard protein isolation protocol. The concentrations of the protein samples were determined by Spectrophotometer (Thermo Scientific, USA) at 595 nm (absorbance). Proteins were separated by gel electrophoresis (10 µg/well) at 125 V for 90 min then transferred to PVDF membrane, incubated in blotto (5.0 g non-fat dry milk and 100 ml of 1 X TBST) at 4°C for 24 h. Membranes were stained with specific primary antibodies (VEGF, Flt-1, Flk-1/KDR, HIF-α) at 1:500 dilution as described by manufacturer and incubated overnight at RT. Membranes were then washed with 1XTBST (5 min each x 3), and incubated with secondary antibody (i.e., Donkey anti-rabbit IgG HRP conjugated, Streptactin HRP) for 1 h at RT, followed by 1 X TBST washes (5 min each x 3), fourth wash with 1 X TBS. Membranes were then covered with limunol-enchancer/peroxidase solution (VWR, USA) and developed. Images were analyzed with ImageJ program (NIH). The specific bands of interest were identified using a standard ladder with known molecular weight (β-actin).

ii). Confocal Immunofluorescence: Experiments were designed to visually identify the specific cell types expressing the proteins of interest, and to complement Western Blot analysis. Paraffin fixed sections were prepared as described previously and incubated with primary antibodies, as described by the manufacturer (Santa Cruz Biotechnology, Santa Cruz, CA). Briefly, after de-paraffinization, non-specific protein bindings were blocked using 10% normal goat serum (NGS, in 0.1 M PBS) (Vector laboratories Inc., USA) at RT for 30

and 45 min for mouse and human tissues, respectively. Tissues were then incubated with appropriate primary antibodies at 0.5 µg/ml (VEGF 164, Flt-1 and Flk-1/KDR) overnight in humid chambers at 4ºC, followed by washes (5 min each x 3) in 0.1M PBS. Control sections were incubated with 10% NGS only. All sections were incubated with fluorescence-tagged secondary antibodies at 0.5 µg/ml for 1 h, followed by washes (5 min each x 3) in 0.1 M PBS. Nucleic acid was counterstained with 5 µM Sytox®Green for 5 min, followed by additional 5 min washes in 0.1 M PBS. Images were obtained using a confocal microscope (Zeiss LSM510, Thornwood, NY).

c). *Gene Expression Studies*: Gene expression analysis was performed using real time PCR to examine the effects of E_2 and mimicked hypoxia on the mRNA expression of VEGF, VEGFRs and HIF-α in the uterine cervix of non-pregnant mice. Gene expression analysis was performed in three steps, as described below.

i). Tissue processing, messenger RNA isolation and quantification: Total RNA was isolated from individual uterine cervices using the RNeasy Mini Kit (Qiagen, Valencia, CA) and purity of each sample was determined using Nanodrop Spectrophotometer (ND-2000, Thermo Scientific, USA). Total RNA was diluted (1.0 µg/9.5 µl) in RNase-free deionized (DI) water, stored at -80°C until processed for reverse transcriptase PCR (RT-PCR).

ii). Reverse transcriptase PCR (RT-PCR): Total RNA from the uterine cervical tissues was reverse-transcribed and amplified in an Eppendorf Thermocycler (Hamburg, Germany) using reagents and protocol from the manufacturer (Applied Biosystems, Foster, CA). Briefly, to generate complementary DNA (cDNA), diluted total RNA were incubated in a water bath for 5 min at 65°C, followed by incubation at RT for 10 min. 9.5 µl of reverse transcriptase master mix was added to each tube, which comprised of: reverse transcriptase

buffer and Magnesium chloride ($MgCl_2$)[2 µl each per RNA (1.0 µg/9.5 µl)], dNTP and RNAse-free water [2 µl each per RNA (1.0 µg/9.5 µl)], RNase inhibitor [0.5 µl per RNA (1.0 µg/9.5 µl)] , and random hexamers [1 µl per RNA (1.0 µg/9.5 µl)]. Lastly, 1.0 µl of MuLV reverse transcriptase was added to each tube. All materials were purchased from Applied Biosystems (Foster, CA). One tube without reverse transcriptase enzyme served as a non-template control (NTC) for DNA contamination. The Thermocycler was programmed at 25°C for 10 min, 42°C for 2 h, 95°C for 5 min, and 4°C. The generated total cDNA was then used to evaluate mRNA levels of the genes of interest, as described below.

iii). Real-time PCR: Relative expressions of VEGF, KDR and Flt-1 were evaluated using real time PCR. Genes of interest (DNA) were amplified using the Applied Biosystems real time-PCR machine (ABI 7300 HT) with the GeneAmp 7300 HT sequence detection system software (Perkin-Elmer Corp), assays and protocol provided by the manufacturer (Applied Biosystems, Foster, CA). Briefly, TaqMan® Gene Expression Assays, which are pre-designed and pre-optimized gene-specific probe sets, were utilized and PCR reactions were set up in 96-well plates (25 µl per well). The reaction components comprised of 1.0 µg/5.0 µl of previously synthesized cDNA, 12.5 µl of 2 X Taqman® Universal PCR Master Mix, 1.25 µl of 20 X Assays-on-Demand TM Gene Mix (e.g., VEGF), and 6.25 µl of real time PCR-grade RNAse-free water. The program was set as follows: 50°C for 2 min and 95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 60 s. The relative expression of the genes of interest were calculated from the threshold cycles with the instrument's software (SDS 2.0) (Applied Biosystems, USA) and normalized against Gusβ.

Statistical Analysis

Data were analyzed using *t* test and ANOVA for multiple comparisons. *p*-values equal to, or less than 0.05 were considered to be statistically significant.

Results

Hypoxia simulation induces morphological changes and regulates mRNA and protein expression of VEGF and its receptors in the uterine cervix of mice

a). *Basic histological changes:* H & E staining performed to explore the basic histological changes in the uterine cervix of mice after mimicking hypoxia using $CoCl_2$ revealed an increase in nuclei staining at 3 h post $CoCl_2$ treatment, signifying an increase in cellular nuclei density, likely from endothelial cells due to an apparent increase in angiogenesis, compared to negative control (Figure 1A-B).

b). *Hypoxia simulation induces changes in expression of HIF-α, VEGF and VEGFRs mRNA and protein in uterine cervix of non-pregnant mice:*

i). Western blot: Levels of HIF-1α, which was used as a positive control protein to determine whether hypoxia was successfully simulated, showed up-regulation in a time-dependent manner following the treatment of the animals with $CoCl_2$, significantly higher than the control at 3 h post injection (Figure 2A), indicating the effectiveness of $CoCl_2$ in simulating hypoxia. The expression of VEGF protein showed significant up-regulation at 30 min post $CoCl_2$ injection, but, thereafter, declined in a temporal fashion, to our surprise (Figure 2B, $p \leq 0.05$). Overall, the protein expression of VEGF receptors were regulated variably; specifically, the up regulation of Flt-1 was most noticeable at 1.5 h post $CoCl_2$ administration with no statistical significance, whereas, expression of KDR protein was

significantly up-regulated at 30 min post $CoCl_2$ administration, followed by a decline to control level ($p \leq 0.05$) (Figure 2C-D).

ii). Confocal Immunofluorescence: Data generated from confocal immunofluorescence were used to supplement data obtained by Western blot analysis. The intensity of VEGF protein expression was found to be more pronounced at 3 h post $CoCl_2$ administration compared to the negative control group (Figure 3A-B). Similarly, the intensity of Flt-1 expression was much greater at 3 h post $CoCl_2$ administration compared to the negative control group (Figure 3C-D). No significant differences were observed between the negative control group and 3 h post $CoCl_2$ administration for KDR expression, consistent with the data generated from Western blot (Figure 3E-F).

iii). Real time PCR*:* Messenger RNA expression of the positive control gene, HIF-1α, surprisingly down-regulated immediately after administration of $CoCl_2$ (Figure 4A, $p \leq 0.05$). The expression of the VEGF gene showed regulation in time dependent manner with levels above control by 3 h post $CoCl_2$ administration (Figure 4B, $p \leq 0.05$). Flt-1 gene expression was found to be significantly higher than control level at 3 h post $CoCl_2$ administration (Figure 4C, $p \leq 0.05$). On the other hand, the gene expression of KDR was down-regulated time-dependently (Figure 4D, $p \leq 0.05$).

E_2 regulates histomorphology of uterine cervix and expression of VEGF and its receptors in non-pregnant mice

a). *E_2 induces changes in the histomorphology of uterine cervix*: Uterine cervices from non-pregnant ovariectomized mice treated with E_2 showed significant changes in tissue weight compared to the control group (Figure 5A-B) however, there were no significant

differences between the treatments (2 mg/kg-8 mg/kg). Hypertrophy of uterine cervical epithelial cells and increased mucous secreting cells were observed (Figure 6A-B).

b). *E_2 regulates expression of VEGF and VEGFRs proteins*:

i). Western blot: Data generated from Western blot shows that E_2 up regulated VEGF ($p \leq 0.05$) and Flt-1proteins in a dose-dependent manner (Figure 7A-B), however the level of VEGF proteins were found to be above the control level only at high dosage ($p \leq 0.05$) . The expression of KDR, on the contrary, was found to be down regulated below control level ($p \leq 0.05$) (Figure 7C).

VEGF and VEGFRs (Flt-1 and KDR) exhibit increased protein expressions in late (day 17) compared to early (day 11) pregnancy. However, surprisingly protein levels of VEGF and Flt-1throughout pregnancy were found to be lower than the control group, i.e., uterine cervix of ovariectomized non-pregnant (data not shown).

ii). Confocal Immunofluorescence: were used to complement data generated from Western blot analysis. VEGF expressions in the negative control group were largely localized in the epithelial and just below it, i.e., sub-epithelial compartments (Figure 8A). Cellular localization of VEGF in E_2-treated (high, 8 mg/kg) animal was more widespread, beyond the epithelial and sub-epithelial compartments, and included stromal cells (Figure 8B). Flt-1 expression was visually noted in both the negative control group and in E_2-treated mice. Intensity of Flt-1 expression was more pronounced in E_2-treated mice, particularly near and on the epithelia (Figure 8C-D). Minimal differences in KDR expression were observed between the negative control group and E_2-treated mice (Figure 8E-F).

iii). Real time PCR: Although differences in VEGF gene expressions between negative control and E_2-treated groups shows no statistical significance, according to the real

time PCR analysis, there was a consistent dose-dependent increase in the expression of this gene. On the contrary, Flt-1 mRNA was below control level and down regulated in dose dependent manner. Level of KDR mRNA was also below control level, and showed no expression pattern (Figure 9A-C).

VEGF and its receptors are expressed in the uterine cervix of non-pregnant women

Data generated from confocal immunofluorescence of human uterine cervix shows presence of VEGF and its receptors (Figure 10A-C). All the three proteins of interest were found to be expressed intensely. However, due to different self-reported phases of the menstrual cycle, there were variations in both the intensity of expression and cellular localization between the subjects. For instance, expression of VEGF was most intense in subject NPO8 and was largely localized in apparent stromal cells (Figure 10A), whereas, expression of Flt-1 and KDR were both expressed in all the women, but their cellular localization was largely confined to epithelial cells (Figure 10B-C). These patterns of cellular localization of VEGF and its receptors in human uterine cervix were comparable to the uterine cervix of ovariectomized non-pregnant mice (Figure 10D-F).

Discussion

The human female reproductive tissues undergo marked changes during gestation and menstrual phases. Supporting and sustaining these tissue changes require an equally dynamic vascular network remodeling that ensures access to essential resources and removal of toxins and metabolic waste products from cells (Andersson et al., 2008). VEGF is the best characterized angiogenic regulator and is the key architect of the vascular network. Our prior studies have characterized the presence and the expression profile of VEGF (164) and its receptors in the uterine cervix of pregnant rodents over the course of pregnancy (Shifren et al., 1996; Mowa et al., 2004; Simon and Einspanier, 2009; Sharma et al., 2011). We do know in other tissues that hypoxia (HIF-1α) caused by cell growth (i.e., tumor cells) and reduction of cellular O_2 supplies (i.e., low blood pressure) is the most potent inducer of VEGF (Calbet, 2003, Yao et al., 2010) and that sex steroid hormones, such as E_2, in the uterus, induces VEGF expression (Mowa et al., 2004). Further, recent studies in the uterus have revealed complex molecular and signaling interplay between E_2 and hypoxia in the induction of VEGF expression (Koos et al., 2005). However, the factors that induce expression of endogenous VEGF and its receptors in the non-diseased uterine cervix are not completely known. Here, we've show that: **i**) hypoxia and E_2 induce expression of VEGF in the uterine cervix of non-pregnant, non-diseased mice; **ii**) the uterine cervix of human expresses VEGF and its receptors (Flt-1 and KDR), similar to mice; **iii**) reveal the cellular pattern of VEGF expression and its receptors (Flt-1 and KDR), in the uterine cervix of rodents. Collectively,

these data shed new insights into the regulation and pattern of VEGF expression in the uterine cervix of rodents and human.

We demonstrate in the present study that chemical simulation or mimicking of hypoxia using $CoCl_2$ alters expression of VEGF and its receptors in the uterine cervix, in a time-dependent manner. Local tissue oxygen tension is tightly regulated, as it plays a critical role in tissue survival and function, under normal physiological conditions. Tissue oxygen levels below normal (normoxia, i.e., $O_2 > 3\%$) lead to hypoxia and, consequently, an increase in the concentration of a hypoxia-associated transcription factor, such as HIF-1α and HIF-2α, which are degraded rapidly in normoxic ($O_2 > 3\%$) conditions by von Hippel-Lindau protein (pVHL) mediated ubiquitination. HIF-1α regulates expression of more than 100 genes, and through signaling cascades it regulates those that are not associated with hypoxia, such as estrogen. $CoCl_2$ interfere the interaction of HIF and pVHL either by disabling the activity of the critical enzyme, HIF hydroxylase (prolyl hydroxylase domain-containing enzymes, PHDs) by binding on its iron binding site, and/or by binding on HIF-α oxygen-dependent degradation domain (ODD) inhibiting the binding of pVHL to HIF-α, even when HIF-α is hydroxylated (Ho and Bunn, 1996; Epstein et al., 2001; Young et al., 2003; Yuan et al, 2003). The effectiveness of $CoCl_2$ in the present study is shown by a time-dependent accumulation of HIF-1α protein; increase in expression of VEGF and proliferation of cells in the tissue, likely to be vascular endothelial cells. However, we are not completely sure of the specific cell type, further study with immunostain with specific endothelial cell markers (i.e., vWF) is necessary to identify these cells. Because the uterine cervix undergoes marked growth over the course of pregnancy, especially in the last two thirds of pregnancy, such pronounced growth is likely to induce hypoxia and, consequently, accumulation of HIF-1α

and expression of VEGF. We do know that hypoxia is generally induced during significant tissue growth or under the influence of growth promoters, such as estrogen and relaxin. For instance, in tumors, when the tissue reaches approximately 0.2-2.0 mm in diameter, without an equivalent change in blood supply, hypoxia is induced, which will then turn on the angiogenic "switch" via induction of HIF-1α (Koh and Powis, 2012). Indeed, our previous studies had shown a close temporal relationship between expression of VEGF/receptors and gestational age (Mowa et al., 2004). Moreover, it is interesting to note that women in Peru, when close to their due date, can intentionally induce the birth process by going to higher altitudes, where oxygen levels are low or conditions are relatively hypoxic (Bonney, 2012). However, more studies are needed to characterize the role of HIF-1α in pregnancy and decipher the molecular mechanisms and pathways that mediate these biological effects in the uterine cervix under physiological and aberrant conditions, such as preterm labor.

Sex steroid hormones, including E_2, are known to differentially alter the expression of VEGF and its receptors in a variety of tissues (Cullinan-Bove and Koos, 1993; Hyder et al., 1996; Hyder et al., 1997; Yoshigi and Keller, 1997; Hyder et al., 1998; Ruohola et al., 1999; Classen-Linke et al., 2000; Hyder al., 2000; Mueller et al., 2000; Nakamura et al., 2000; Stoner et al., 2000; Bogin and Degani, 2002; Buteau-Lozano et al., 2002; Stoner et al., 2004). While E_2 promotes expression of VEGF in the uterus and mammary tumors in the female rats (Nakamura et al., 2000), regulation of VEGF by E_2 in some tissues is much more complex and appears to be context-dependent. For instance, whereas, E_2 induces VEGF gene expression in Ishikawa and MCF-7 breast cancer cells (Ruohola et al., 1999; Mueller et al., 2000; Buteau-Lozano et al., 2002), it (E_2) decreases VEGF gene expression in HEC1A endometrial cancer cell lines, even though they are also estrogen receptor (ER)-positive

(Stoner et al., 2000). Although ER and VEGF and its receptors have been described previously in the uterine cervix and appear to have a temporal relationship, until now, very little was known whether there was a cause-and-effect relationship between the two factors. Here, we have shown that exogenous E_2 induces expression of VEGF mRNA and protein in the uterine cervix in a dose-dependent manner. Our present findings correlate with studies that used rodent uterus, Ishikawa and MCF-7 breast cancer cells, as cited above (Ruohola et al., 1999; Mueller et al., 2000; Buteau-Lozano et al., 2002).

For now, it is not clear exactly how E_2 induces expression of VEGF and its receptors in the uterine cervix and how this relates to uterine cervical events, notably CR. It is, however, intriguing to note some striking similarities and overlap between the biological effects of VEGF with another E_2 responsive gene whose actions on the uterine cervix have been extensively characterized, namely relaxin. Both relaxin and VEGF: **a**) are responsive to E_2; **b**) induce proliferation of uterine cervical epithelial cells and expression of endothelial nitric oxide synthase (eNOS) in the uterus, dose-dependently (Bani-Sacchi et al., 1995; Unemori et al., 1999; Dschietzig and Stangl, 2003); **c**) reduce tissue tensile strength in rodents and; **d**) exert similar vascular effects (normal and aberrant), such as vasodilation (Bani and Bigazzi, 1984; St-Louis and Massicotte, 1985). For instance, in women with scleroderma administered continuous subcutaneous infusion of relaxin over 24 weeks, relaxin mimics typical VEGF-like vascular physiological effects, described in human pregnancy, particularly increased blood volume, flow and angiogenesis (Seibold, 2002). Notable side effects reported included hyper-menorrhagia (excess bleeding), which is likely due to abnormal vascular permeability or vessel leakage, another signature characteristic of aberrant VEGF concentration (Seibold, 2002). Lastly, a recent report concluded that the

primary role of relaxin mainly involves regulation of body fluids and circulation hemostasis, roles that are comparable to VEGF action (Dschietzig and Stagl, 2003).

The effects of VEGF are known to be mediated by its two receptors, Flt-1 and KDR, which have been localized previously in the uterine cervix by our group. Here, we show that the mRNA and protein levels of both receptors are variably regulated by simulating hypoxia and exogenous E_2. It is not clear which receptor is likely to mediate the biological effects of VEGF that have been so far reported, e.g., epithelial proliferation, immune cell recruitment, tensile strength alteration and others. However, we do know that in other tissue types VEGFR2/KDR/Flk-1 is the main regulator of VEGF under normal physiological conditions; it has been shown to mediate several biological processes such as proliferation, increase in vascular permeability, cell migration and inhibition of cell apoptosis. On the other hand, VEGFR1/Flt-1 is known to have no direct cellular effect, but leads to increased expression of proteases (i.e., plasminogen activator/ inhibitor-I) resulting in degradation of extracellular matrix structure, monocyte migration and sequestration of excess VEGF by the soluble form of Flt-1 (Roskoski, 2007). Lastly, our present study shows presence of VEGF and its receptors in the stromal and epithelial cells of the uterine cervix in human, i.e., non-pregnant women with self-reporting phases of menstrual cycles. The similarities in the cellular expression of these molecules between rodents and human could potentially imply existence of comparable mechanisms and biological actions in these two species, and, possibly, suitability of rodents as models for studying VEGF's role in CR.

We conclude that levels of VEGF are altered by hypoxia simulation and exogenous E_2 in a time- or dose dependent manner in the mice; are present and variably expressed in the uterine cervix of non-pregnant women and that they are synthesized by multiple cell types of

both mice and human uterine cervix. These findings may shed new insights into deciphering the role of VEGF and its underlying mechanism in CR, as it relates to pre-term labor, an obstetrical problem affecting both mothers and babies.

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Tables

Table 1: *Backgrounds of human subjects from which uterine cervical tissues were obtained:* Uterine cervical tissues were harvested from four women, with self-reporting phases of menstrual cycle, immediately after total abdominal hysterectomy. Harvested tissues were then processed and imbedded in paraffin blocks and sections were cut and analyzed for histology and confocal immunofluorescence (source of tissues; generously provided by Dr. Dilly Anumba).

SUBJECTS REFERENCE NUMBER		**NP05**	**NP06**	**NP07**	**NP08**
BACKGROUND	Age (years)	35	40	44	41
	Smoking status	No	No	No	Yes
	Last Cervical Smear in 5 years	Yes	Yes	Yes	Yes
	Last smear normal	Yes	Yes	Yes	Yes
	Menopausal Symptoms	No	No	No	No
	Hysterectomy type	Total	Total	Total	Total
	Surgery indication				
	a.*Menorrhagia*	Yes	Yes	Yes	Yes
	b *Malignant*	No	No	No	No
	c *Chronic Pelvic Pain*	Yes	Yes	No	Yes
	d *Other benign*	No	No	No	No
	Menstrual Phase (secretory/menstrual)	S	M	S	M
	Hormonal treatment, last 6 weeks	No	No	No	No
	Number, previous pregnancies	3	2	1	1
	Number, miscarriages	1	0	0	0
	Delivery type (Vaginal/caesarian)	C	V	C	V
	Vaginal infection, last 6 weeks	No	No	NA	No

Figure 1

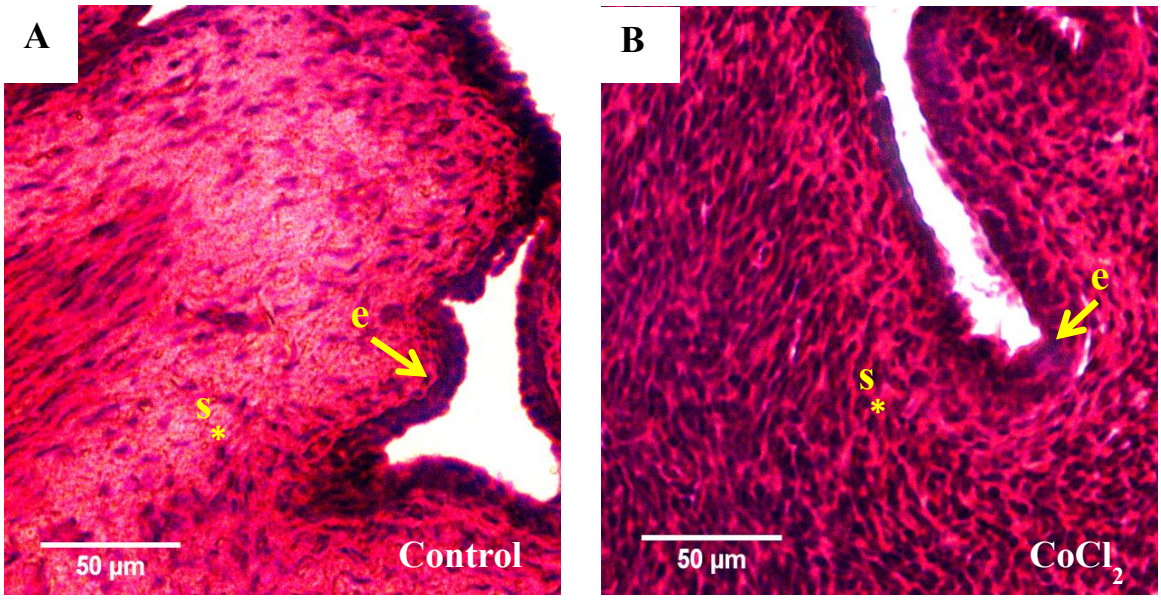

Figure 1: *Effects of $CoCl_2$ on histomorphology of mice uterine cervix:* H&E staining were performed on uterine cervix of mice treated with $CoCl_2$. **A)** Negative control group (Left column, 0.9% NaCl only); **B)** $CoCl_2$-treated group (Right column, 3 h treatment, 38.95 mg/kg body weight) (20x) "**B**" compared to "**A**" shows increased density in cellular nuclei (dark blue dots, *), indicative of cell proliferation, most likely endothelial cells, which occurs during angiogenesis. e= epithelia; *= stromal cells.

Figure 2

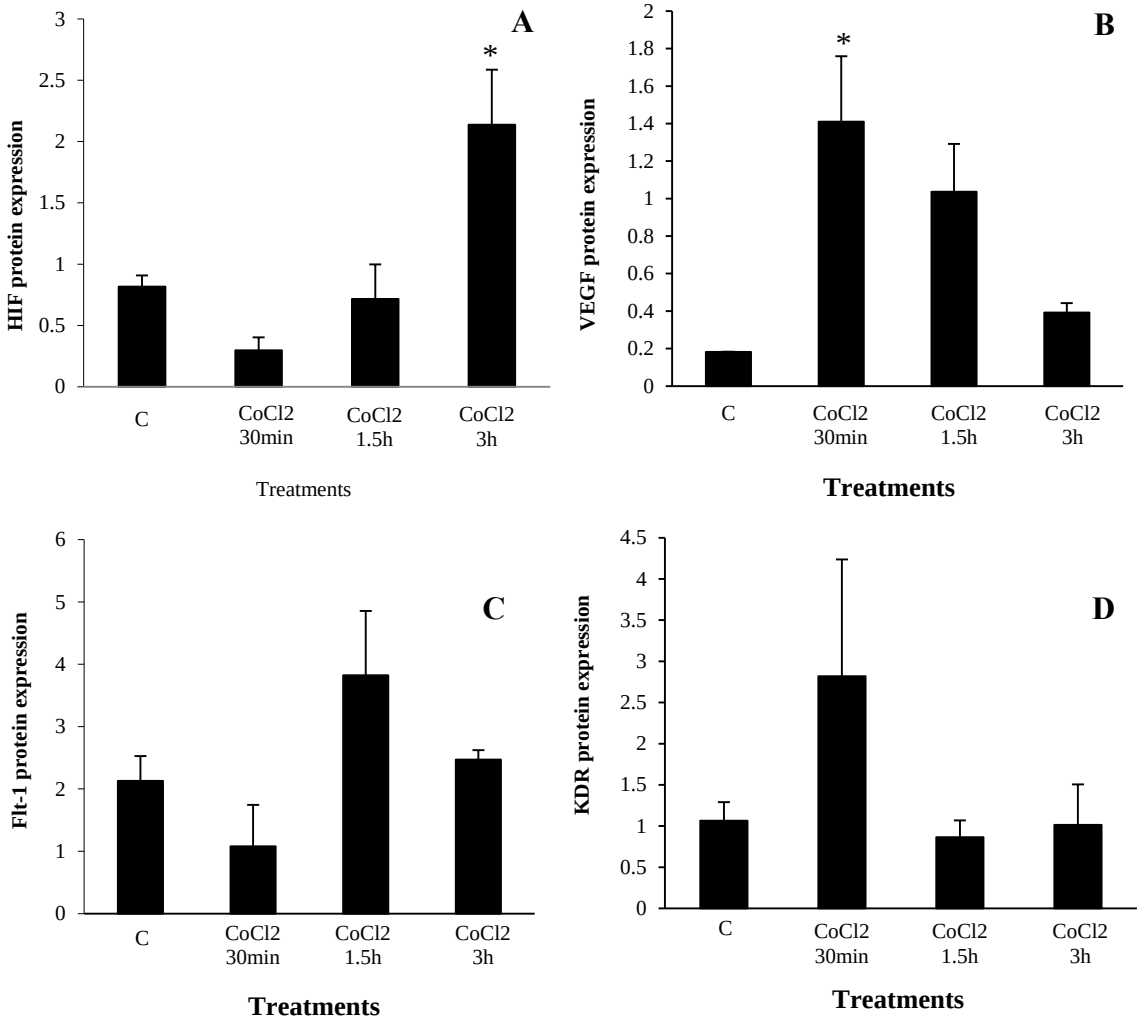

Figure 2: *Effects of $CoCl_2$ on expression of VEGF, KDR and Flt-1 proteins in the uterine cervix of non-pregnant mice, as revealed by Western Blot analysis:* Mice were either treated with $CoCl_2$ (38.95 mg/kg body weight) for 30 min, 1.5 h and 3 h, respectively. Levels of HIF-1α showed up-regulation in a time-dependent manner however it was only significantly higher than the control at 3 h post injection (**A**). The expression of VEGF protein showed significant up-regulation at 30 min post $CoCl_2$ injection, but, thereafter, declined, in a temporal fashion (**B**). The up regulation of Flt-1 was most noticeable at 1.5 h post $CoCl_2$ administration with no statistical significance (**C**), whereas, expression of KDR protein was significantly up-regulated at 30 min post $CoCl_2$ administration, followed by a decline to control level (**D**).

* = $p < 0.05$; n=3.

Figure 3

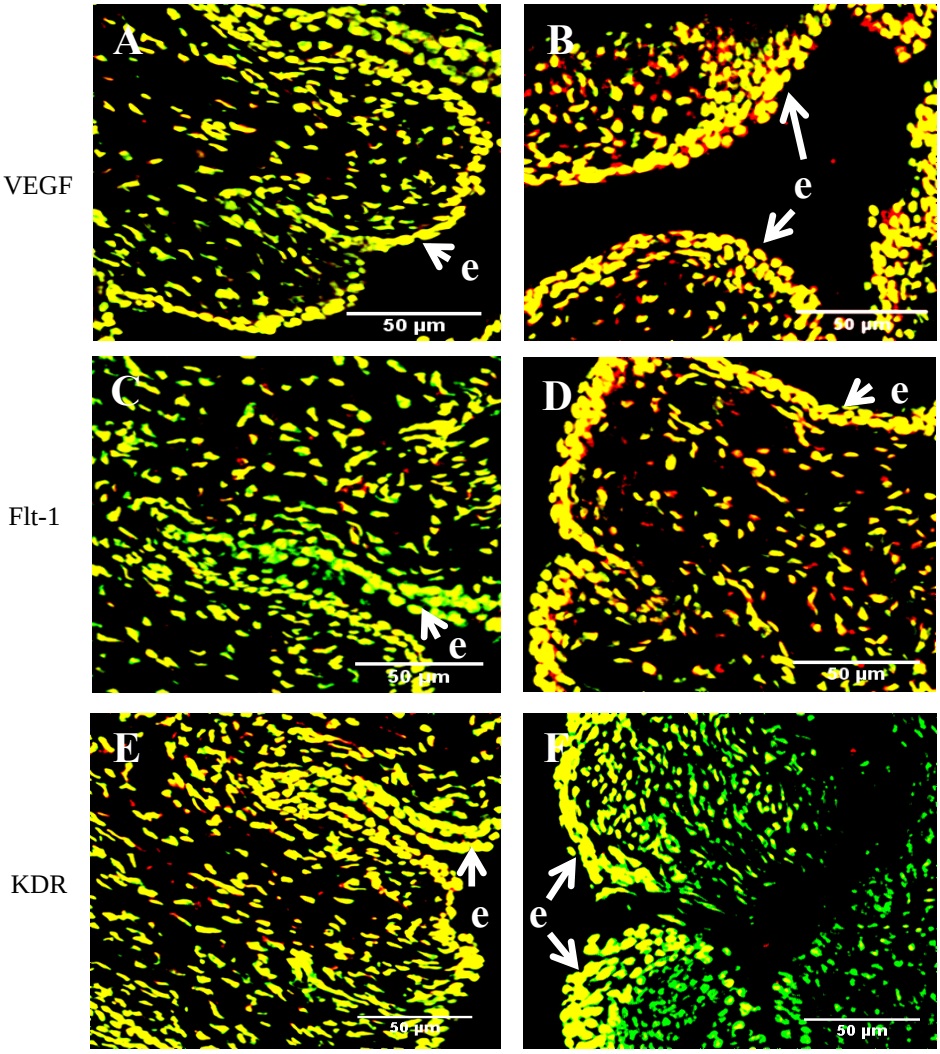

Figure 3: *Effects of $CoCl_2$ on expression of VEGF, Flt-1 and KDR proteins in the mice cervix, as revealed by confocal immunofluorescence:* Uterine cervices of mice treated with $CoCl_2$ were immunostained with different primary antibodies of target proteins (red fluorescence) and counterstained with Sytox®green, which is displayed as green fluorescence in the cellular nuclei. Images on the left column (**A**, **C**, **E**, x40) were from 0.9% NaCl only-treated animals (negative control group) and those on the right column were from $CoCl_2$-treated animals (**B**,**D**, **F**, x40) (3 h, 38.95 mg/kg body weight): $CoCl_2$ treatment induced a more pronounced expression of the target proteins (VEGF: **A** vs. **B**; Flt-1: **C** vs. **D**) compared to control, except for KDR (**E** vs. **F)** in both stromal and epithelial cells;. e = epithelia; red/yellow = cells with positive protein expressions; green = cellular nucleic.

Figure 4

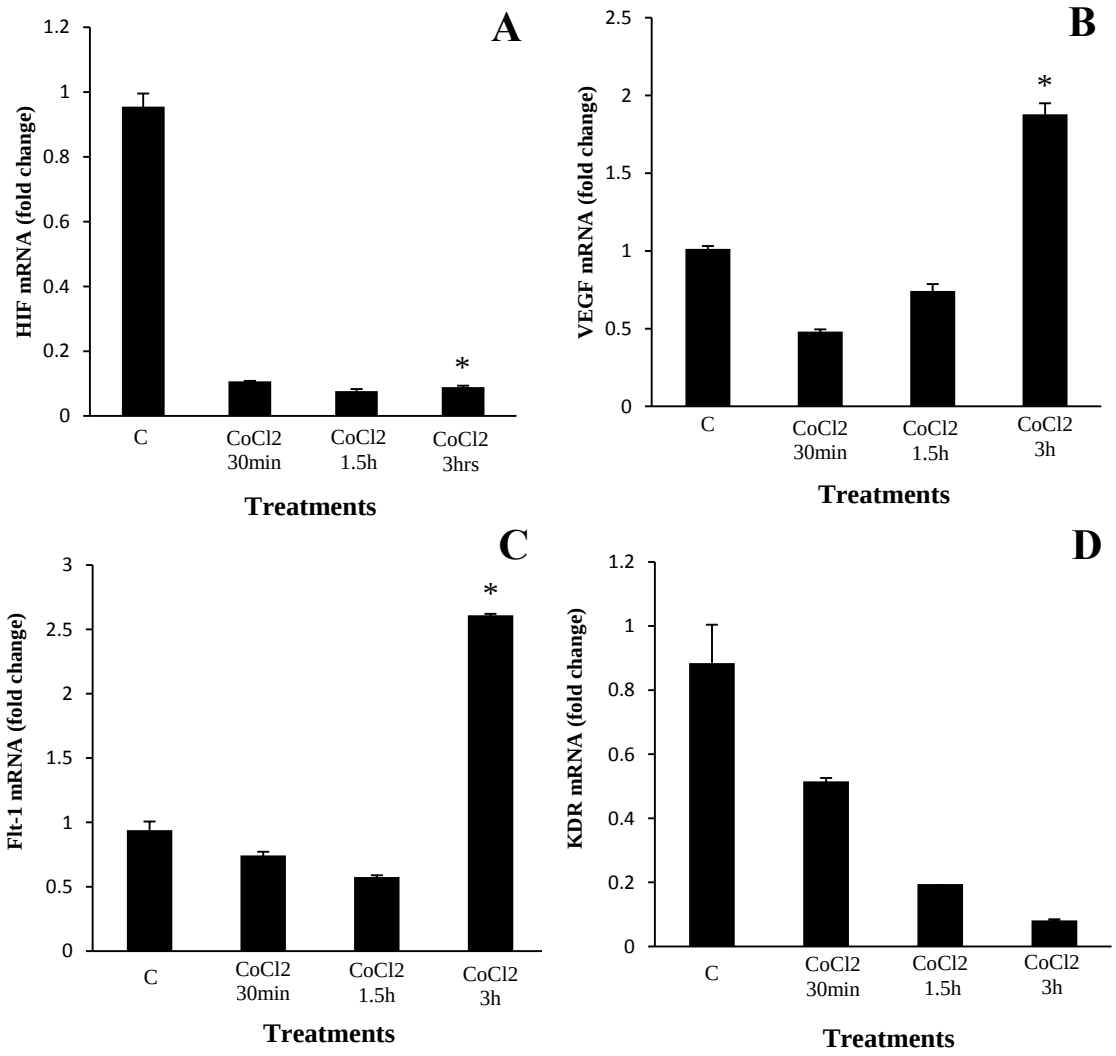

Figure 4: *Effects of $CoCl_2$ on mRNA expression of HIFα, VEGF, KDR and Flt-1 in the uterine cervix of non-pregnant mice, as revealed by real-time PCR:* Treatment of mice with $CoCl_2$ (38.95 mg/kg) for 30 min, 1.5 h and 3 h, down-regulated mRNA expression of HIF-α immediately after administration of $CoCl_2$ (**A**), VEGF (**B**) and Flt-1 (**C**) in the cells of the uterine cervix, in a time-dependent fashion with levels above control by 3 h post $CoCl_2$ administration. The gene expression of KDR was down-regulated time-dependently except that of KDR (**D**). * = $p < 0.05$; n=3.

Figure 5

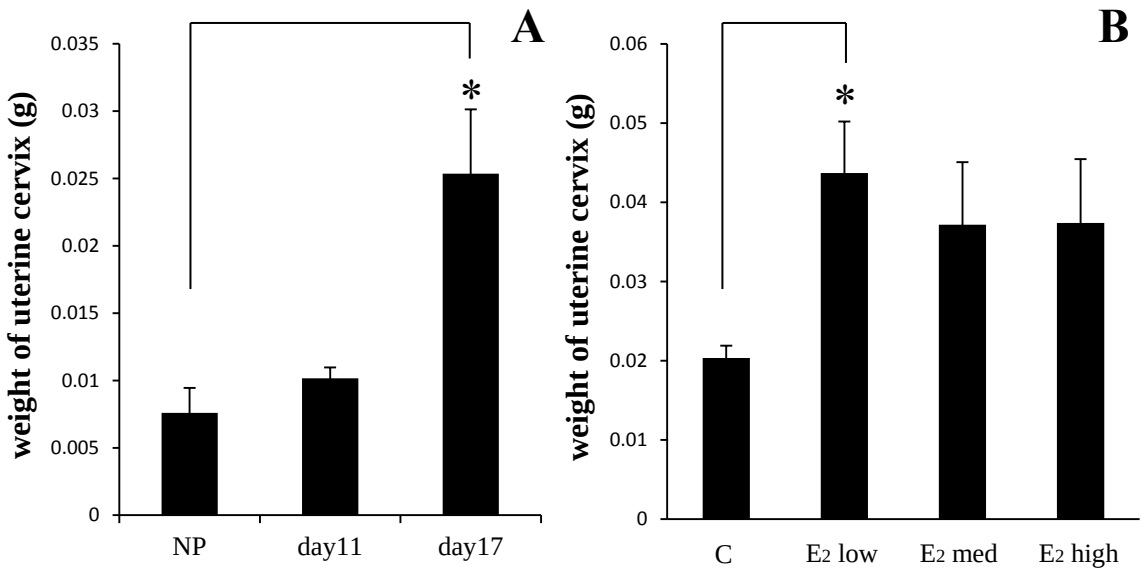

Figure 5: *Effects of pregnancy and 17β-estradiol on wet weight of mice uterine cervical tissue:* The wet weight (g) of uterine cervix increased between early and late pregnancy (days 11 and 17 of gestation, respectively) (**A**) and E_2-treated (2 mg, 4 mg, and 8 mg/kg body weight) (**B**) mice compared to their respective controls however, there were no significant differences between the different E_2 treatment dosages. NP=non-pregnant; C=negative control. * = $p < 0.05$; n=3.

Figure 6

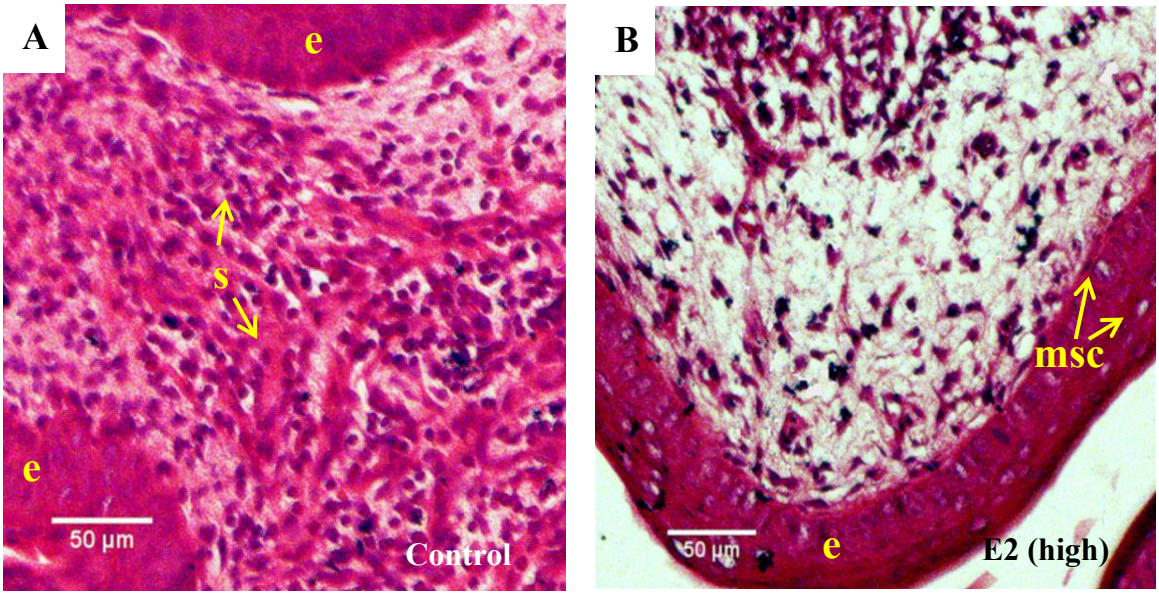

Figure 6: *Effects of 17β-estradiol on the histomorphology of mice uterine cervix* Treatment of mice with E_2 induced uterine cervical tissue edema, demonstrated here as "dispersed" or less dense cell nuclei, and also increase in goblet cells were observed (**B**)**(**8 mg/kg body weight) compared to negative control group (**A**)(0.9% sesame oil only). e = epithelia; s = stromal cells.

Figure 7

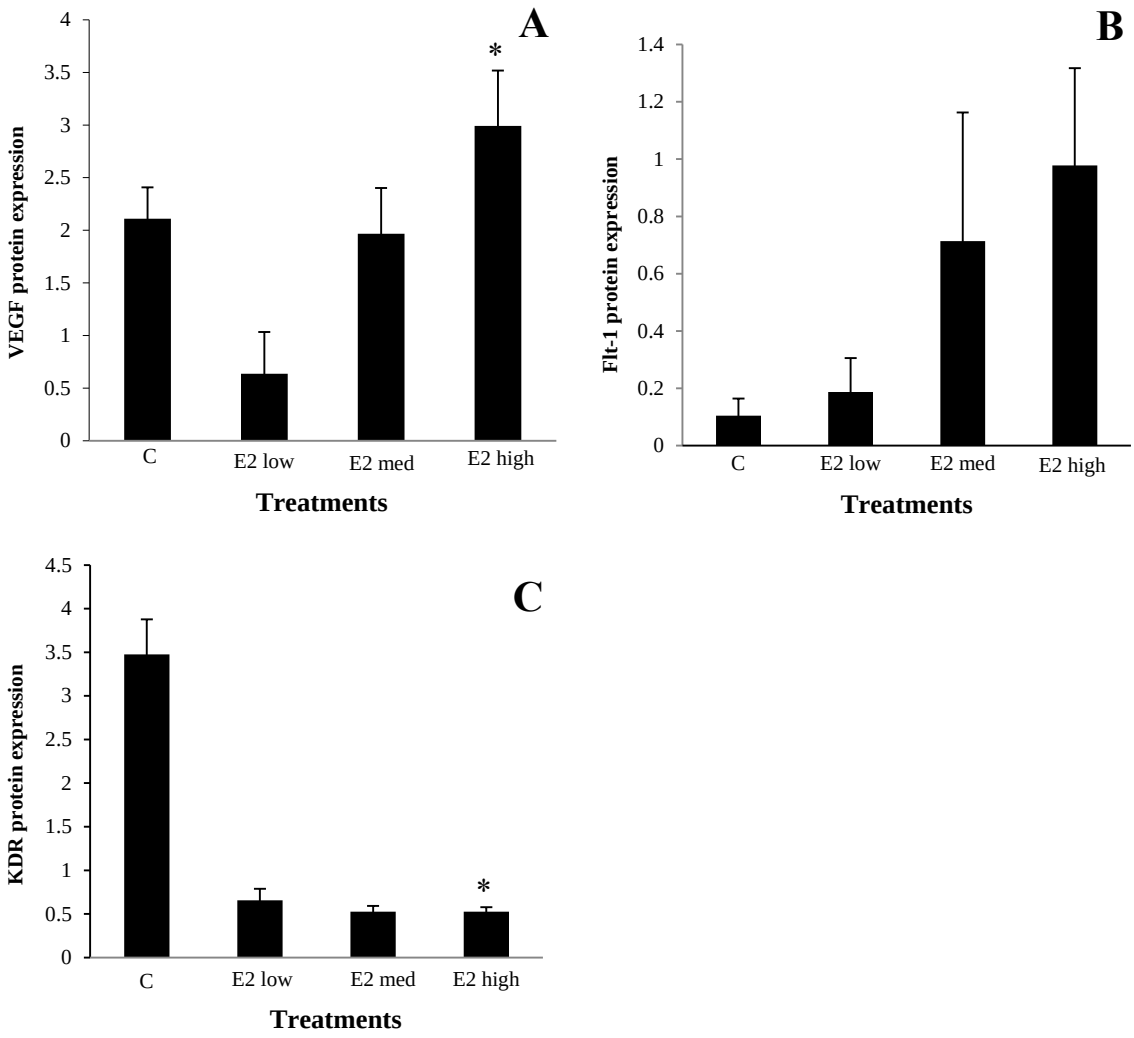

Figure 7: *Effects of 17β-estradiol on expression of VEGF, KDR and Flt-1 proteins in the uterine cervix of non-pregnant mice, as revealed by Western Blot:* E_2 altered protein expression of VEGF however the level of VEGF proteins were found to be above the control level only at high dosage (**A**)(8 mg/kg body weight) while Flt-1showed up-regulation in dose-dependent manner (**B**)(2 mg, 4 mg and 8 mg/kg body weight) whereas KDR was down regulated below the control level (**C**). * = $p < 0.05$; n=3.

Figure 8

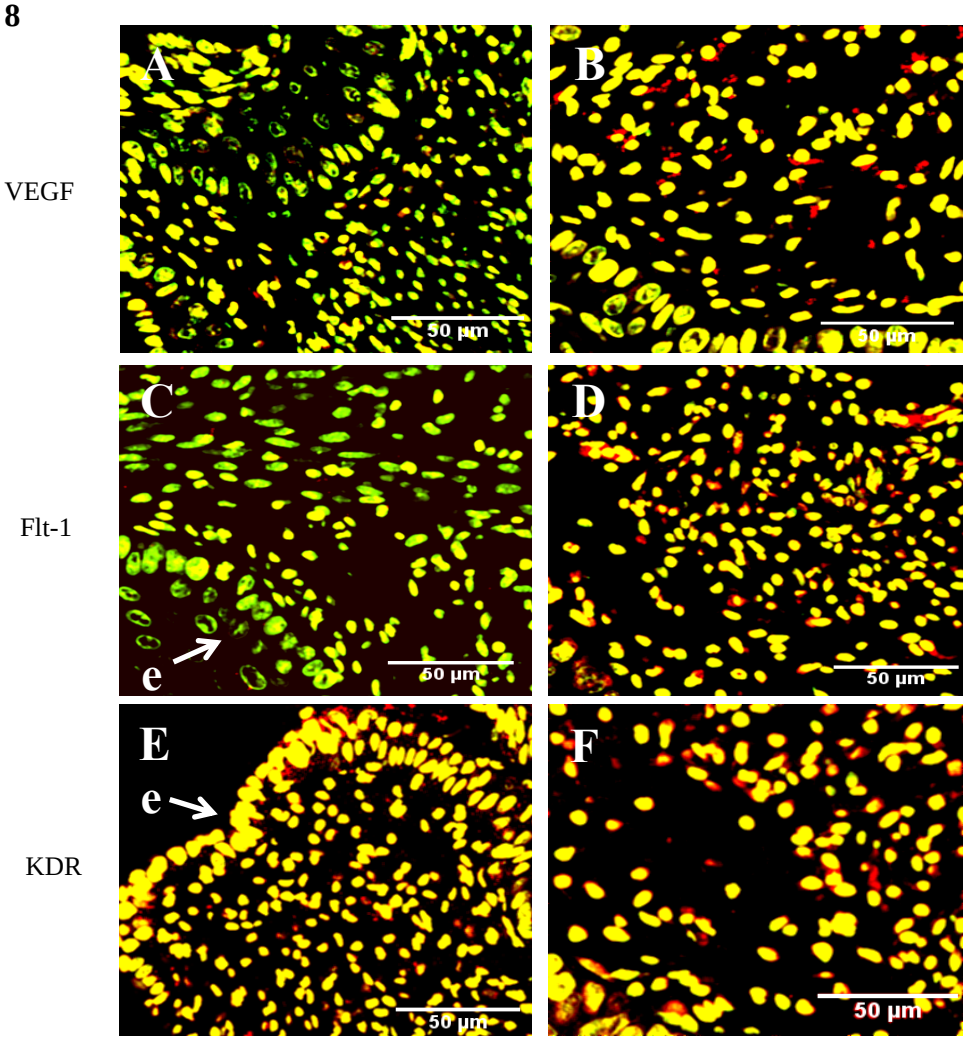

Figure 8: *Effects of 17β-estradiol on expression of VEGF, Flt-1 and KDR proteins in the mice uterine cervix, as revealed by confocal immunofluorescence:* Uterine cervices of mice treated with E_2 were immunostained with different primary antibodies of target proteins (red fluorescence) and counterstained with Sytox®green, which is displayed as green fluorescence in the cellular nuclei. E_2 was found to alter protein expression of VEGF (**A**) and its receptors (Flt-1, **B**; KDR, **C**, respectively) (8 mg/kg body weight) in uterine cervical tissue of mice. Both expression of VEGF and Flt-1 were up-regulated, whereas, KDR was down-regulated. Images on the right column (**B, D, F**) are from animals treated with E_2 and those on the left column (**A, C, E)** are from the negative control group (sesame oil only). e = epithelia; red/yellow = cells with positive protein expressions; green = cellular nucleic.

Figure 9

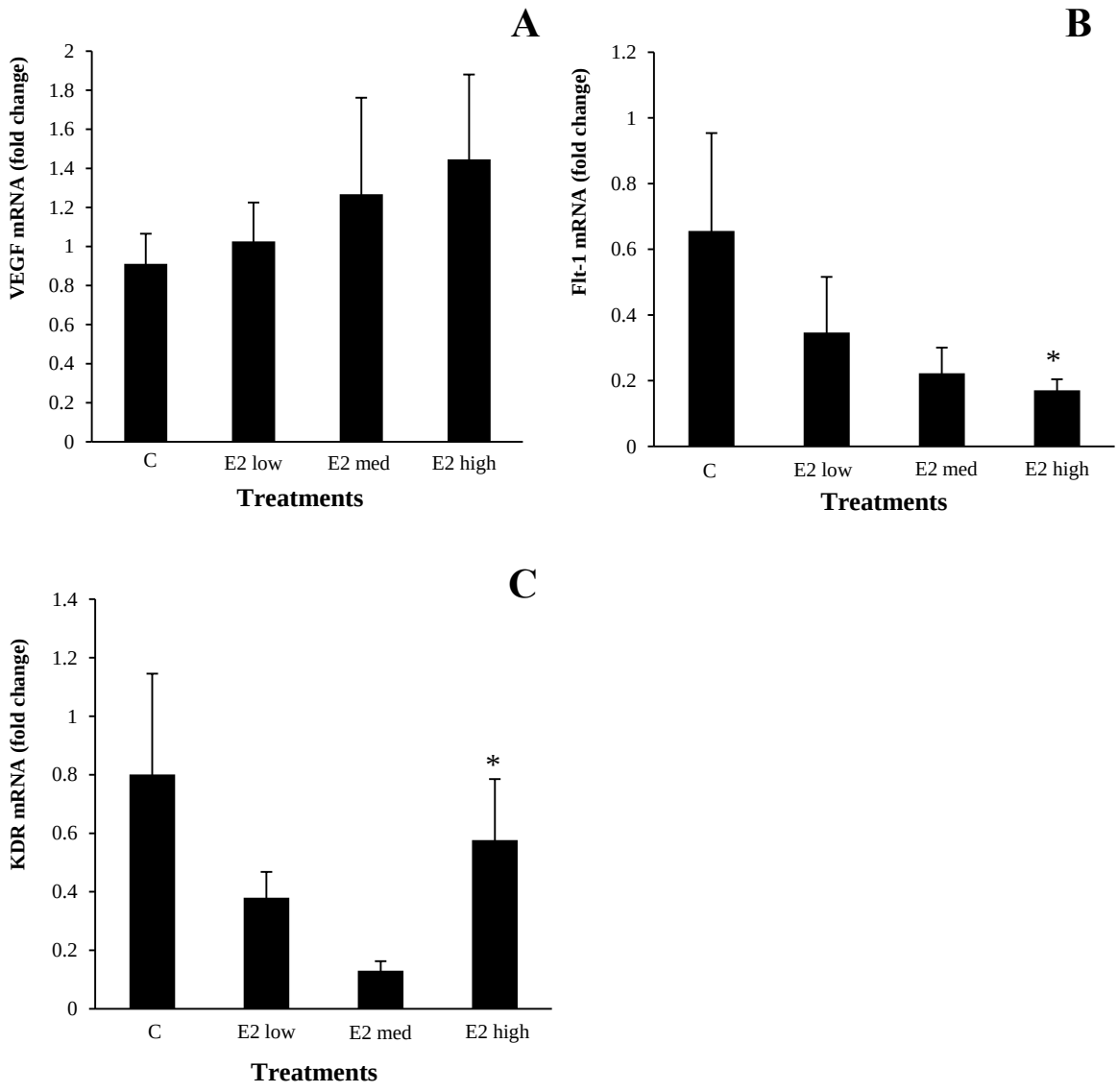

Figure 9: *Effects of 17β-estradiol on mRNA expression of VEGF, KDR and Flt-1 in the uterine cervix of non-pregnant mice, as revealed by real time PCR:* E_2 treatment altered mRNA expression of the target genes differentially (E_2: 2 mg, 4 mg/kg and 8 mg/kg body weight): **A**) It up regulated VEGF mRNA expression dose-dependently (**A**); down regulated Flt-1 mRNA expression (**B**); and had a varied influence with no expression pattern on KDR mRNA expression (**C**). * = $p < 0.05$; n=3.

Figure 10

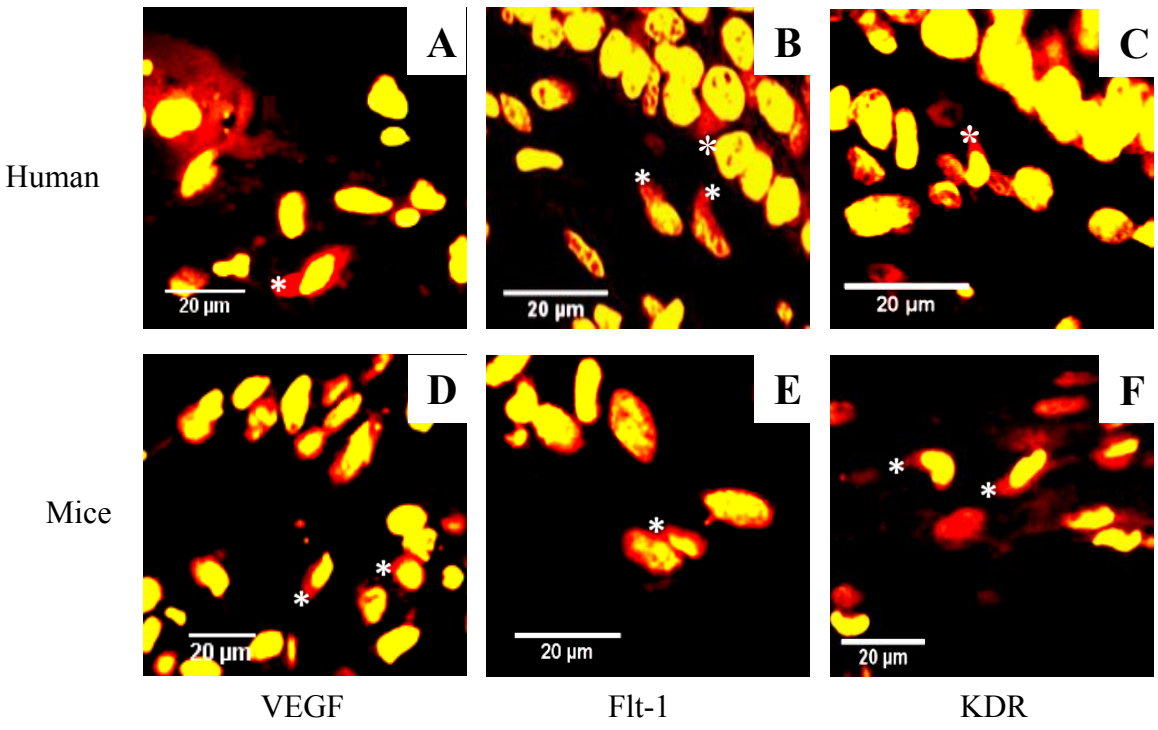

Figure 10: *VEGF and VEGFRs protein expressions in the uterine cervix of human and mice:* Confocal immunofluorescence of VEGF and its receptors (Flt-1 and KDR) were localized in the uterine cervical cells of non-pregnant human (Top row, Figure **10A-C**) and mice (Bottom row, Figures **10D-F**). Red/yellow = cells with positive protein expressions; green = cellular nucleic staining

Biographical Sketch

Takako Ohashi currently resides in Boone. N.C., with her son, step children and her husband. She was born in Tokyo, Japan, on November 09, 1978. She attended several schools in Japan and the US, including: **a**) Dai-yon elementary school in Oume-shi, Tokyo, **b**) Zen-gou elementary school in Yabuki-machi, Fukushima, **c**) Dai-ichi elementary school and **d**) Nishibukuro junior high school in Sukagawa-shi, Fukushima, **e**) Hardin Park elementary school, and graduated from Watauga high school in Boone, N.C. in 1997. The following autumn, she enrolled at Appalachian State University as a Music performance major, but later withdrew from the program due to the birth of her son. In the fall of 2004, she re-enrolled at Appalachian but this time as a Biology major and was awarded the Bachelor of Science in May 2010. A year later, she applied for the graduate program in Biology, was accepted to begin her studies in fall 2011, and was awarded a teaching assistantship in spring of 2012. Her graduate thesis focused on characterizing the expression of vascular endothelial growth factor (VEGF) and its receptors in the uterine cervix of human and how estrogen and hypoxia regulates their (VEGF and receptors) expression in mice uterine cervix, under the guidance of Dr. Chishimba Nathan Mowa, with whom she has worked since 2008. The Master of Science was awarded in May 2013, and she plans to pursue a career in health care and or biomedical research.